GAF Domains: Cyclic Nucleotides Come Full Circle

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Hormone signals are commonly converted at the cell membrane to second messengers that propagate the signal within the cell. Cyclic adenosine 3′,5′-monophosphate (cAMP) was the first intracellular messenger to be discovered. This 1957 discovery by Earl Sutherland established a paradigm that has guided research into receptor signaling ever since. The discovery of cAMP as a second messenger raised the obvious question as to whether the other ubiquitous biological purine, guanine, might also have a cognate cyclic nucleotide second messenger. This question was answered in 1963 with the discovery of cyclic guanosine 3′ 5′-monophosphate (cGMP) in urine. In subsequent years, the fields of cAMP and cGMP signaling have developed hand in hand. The enzymes and binding domains responsible for the synthesis, breakdown, and recognition of the two cyclic nucleoside monophosphate (cNMP) messengers are homologous and closely related. New structural (*1*) and functional (*2*) studies of one class of cNMP binding domain, the GAF (cyclic GMP, adenylyl cyclase, FhlA) domain, reinforce the concept of homology throughout the cAMP and cGMP pathways.

The cNMPs are synthesized from their cognate nucleoside triphosphates by a superfamily of cyclases that have either one or two active sites located at the interface between two catalytic domains (*3*-*7*). The catalytic domains are identical to each other in the case of homodimeric adenylyl cyclases (ACs) and guanylyl cyclases (GCs) found in many single-celled eukaryotes and in mammalian transmembrane GC receptors. The two domains are homologous but nonidentical; for example, compare the two domains located within the monomeric mammalian ACs to the heterodimeric soluble GCs that are receptors for nitric oxide (NO) signaling. Specificity for the cNMP is determined by two side chains in the purine-binding pocket of one of the catalytic domains that interact with the key 1, 2, and 6 positions of the purine ring. The specificity can be swapped for guanine (G) to adenine (A) by replacing the Glu and Cys of the GC with the corresponding Lys and Asp of the AC (*8*, *9*). The reverse specificity swap is complicated by the role of a main-chain carbonyl in the AC in interacting with the adenine N6 (*9*). The cNMP phosphodiesterases (PDEs) that cleave the cyclic bond are similar to the cyclases in being a specialized family but differ in that some of these enzymes have mixed specificity for both cAMP and cGMP (*10*-*12*). Further, in some cases, a nonsubstrate or poor substrate cNMP will bind to the active site and inhibit activity with respect to the better substrate. The structure of the catalytic core of PDE4, a cAMP-specific phosphodiesterase, has been determined in the absence of substrate, and the substrate complex has been modeled (*13*). A Gln has been proposed to mediate cAMP specificity, but the picture is not yet as clear as for the cyclases.

The parallels between cAMP and cGMP signaling continue to hold for the regulatory domains [referred to in databases as the cyclic nucleotide-binding (CNB) domain (http://smart.embl-heidelberg.de)] of the most prominent intracellular receptors of cNMP signaling: the cAMP- and cGMP-dependent protein kinases PKA and PKG, the cNMP-gated ion channels, and the cAMP-activated Rap exchange factor Epac. Collectively, this family has members that are specific for one or another cNMP or for both at once. The CNB domain is one of the most ancient elements in cNMP signaling. cAMP is an ancient hunger signal present in all kingdoms of life, although not all of the molecules and symmetries mentioned above are conserved in the most ancient organisms. cAMP signaling is widespread in bacteria, where it is best known for its role in gene regulation through the cAMP-activated catabolite gene activator protein CAP (*14*). cGMP signaling does not occur in bacteria as far as is known, and the cGMP-binding variants of the CNB are only known in the eukarya. Structures have been determined for the CAP (*15*) and the PKA regulatory R subunit complexed with cAMP (*16*). A Thr and an Asp or Glu residue have been proposed as determinants for cGMP binding (*17*, *18*). The PKG regulatory domain contains a Glu, and the cGMP-gated (CNG) ion channel contains an Asp that are predicted to specifically interact with the N1 and N2 positions on guanine, much as seen for guanine recognition by GCs and heterotrimeric GTP-binding proteins.

The GAF (cyclic GMP, adenylyl cyclase, FhlA) domain is a relative newcomer to the domainology of cNMP signaling. It had been appreciated since about 1990 that the NH_2-terminal regulatory domains of certain cNMP phosphodiesterases (PDEs 2, 5, and 6) are allosteric binding sites for cGMP [reviewed in (*10*, *12*)]. These NH_2-terminal sites are noncatalytic and are found in PDEs that hydrolyze cAMP as well as cGMP. Their sequences differ from the CNB domain family. It was not until 1997 that Ponting and Aravind recognized that these regulatory domains comprise a small subset of a much larger superfamily of signaling and sensory domains, the GAF domains (*19*). The crystal structure of a GAF domain from an uncharacterized protein YKG9, coded by the genome of *Saccharomyces cerevisiae*, showed that the GAF domain fold is like that of another ubiquitous signaling and sensory domain, the PAS (Per, Arnt, Sim) domain (*20*). The YKG9 GAF domain does not bind cGMP, and its physiological ligand is not known. The structure of the PDE2 two-GAF-domain regulatory domain has now been determined in complex with cGMP, showing how the cGMP-binding class of GAF domains recognizes its ligand (*1*). The ribose and cyclic phosphate moieties are deeply buried in the domain. The specificity-determining edge of the guanine ring (positions N1, N2, and O6) is buried to a lesser extent. O6 and N1 of the edge interact with the main-chain NH and the side chain of an Asp that is conserved in all known cGMP-binding GAF domain sequences. N2 has a water-mediated interaction with a conserved

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Thr side chain. The observations that one of the key interactions with a potential specificity determinant, N2, is water-mediated and that all of the interactions are close to the protein surface suggest that discrimination between cAMP and cGMP might not be absolute. Indeed, cAMP binds only ~11-fold more weakly to the PDE2 GAF domain than does cGMP. Other GAF domains may have more stringent specificity, and it will be interesting to compare their structures when they become available.

Until very recently, the GAF domain seemed to stand apart from the other protein modules of cyclic nucleotide signaling. Unlike the cyclase and phosphodiesterase catalytic domains and the CNB domain, it seemed to participate only in cGMP, but not cAMP, signaling. Now the symmetry between the two messengers has been restored by the fortuitous identification of a cAMP-binding GAF domain in an AC of the cyanobacterium *Anabaena* by Joachim Schultz and his co-workers (*2*). Their study began with the characterization of cyaB1, one of six ACs encoded by the genome of *Anabaena* sp. PCC 7120. *Anabaena* cyaB1 contains a single COOH-terminal cyclase catalytic domain of the type that is active as a homodimer and two GAF domains and a PAS domain in its NH_2-terminus. The key observation was made that enzyme activity rapidly increased over time. The activity increase was attributed to a positive feedback loop initiated by the production of cAMP by the enzyme. The effect could be reproduced by the addition of exogenous cAMP. Mutational analysis of the GAF domain showed that it was the second GAF domain, GAF-B, that was responsible for the activation.

How does the cyaB1 GAF-B bind cAMP? The structure of the cGMP-binding GAF-B of PDE2 shows how the common ribose and cyclic phosphate moieties are recognized, together with the common hydrophobic features of the purine ring (Fig. 1). Beavo and co-workers proposed an 11-residue fingerprint sequence for cGMP binding (*1*). Eight of these 11 residues in cyaB1 fit this cGMP-binding profile. These positions are involved in recognition of moieties common to both cAMP and cGMP. Of the three positions that do not fit the profile, two (the sequence FD, positions 2 and 3) are potentially involved in specific purine recognition. The D in the motif corresponds to Asp^{439} in PDE2. The side chain of this Asp hydrogen bonds to the guanine N1, and it is the only residue in the binding site with an obvious role in discriminating guanine versus adenine. In cyaB1 GAF-B, the FD sequence is replaced by AA. This leaves the cyaB1 GAF-B with no acceptor for a hydrogen bond from the protonated N1 of guanine, which is consistent with a preference for adenine. This sequence difference alone does not fully account for the specificity differences between the two classes of GAF domains. There is no obvious candidate residue in cyaB1 that could specifically hydrogen-bond to the 1, 2, or 6 positions of the cAMP adenine. Furthermore, the PDE2-GAF-B structure shows that the main-chain NH of Asp^{439} donates a hydrogen bond to the O6 of guanine. This main-chain hydrogen bonding arrangement would be incompatible with binding to the N6 of adenine. One postulates that the main-chain conformation is different in the cyaB1 GAF domain, perhaps because of surrounding sequence differences, or that cAMP binds in a somewhat different orientation.

These differences suggest that the evolution of two cyclic nucleotide specificities was not a trivial matter of changing a few side chains. It must have involved a significant number of changes distributed throughout the surrounding structure. The divergence between the two types of GAF domains is just one facet of the remarkable diversity of the GAF domain family as a whole. GAF domains were only recognized as late as 1997 precisely because their sequences are so diverse that the relationships can only be detected by sensitive multiple alignment methods. On a pairwise basis, the sequence homology between two GAF domains is in many cases negligible. The diversity of the sequences is likely to be mirrored in a great diversity of small-molecule ligands for various GAF domains. The discovery of a cAMP-binding GAF domain increases the repertoire of known GAF ligands considerably, from two to three, but what we know is still just a drop in the bucket.

Although in some respects GAF domains are so diverse that they appear to have little in common with each other, some of their fundamental regulatory properties appear to be conserved. Schultz and co-workers created a chimeric cyaB1 in which its two GAF domains were replaced by those of rat PDE2. Remarkably, the design worked and the chimera was activated by cGMP. Ligand binding to GAF domains leads to allosteric effects on the GAF domain-containing enzymes, but the effects

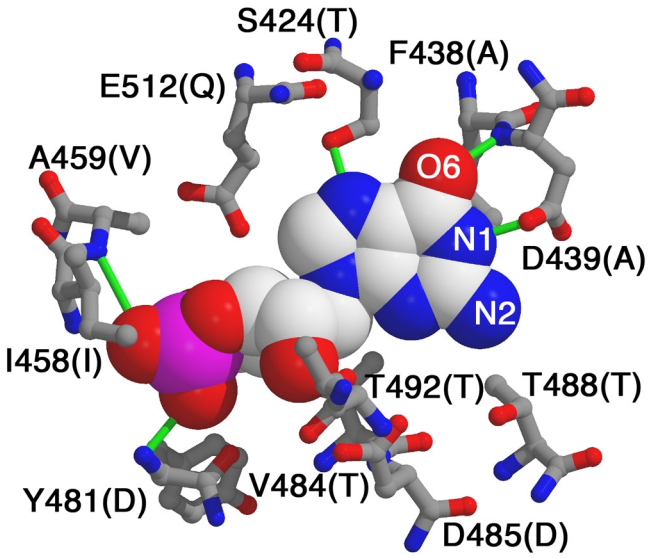

Fig. 1. cGMP bound to the GAF-B domain of PDE2, based on the Protein Data Bank (PDB) coordinates 1MC0 (*1*). The cGMP is shown in a space-filling representation, and the 11 fingerprint residues for cGMP specificity are shown in a ball-and-stick representation. The residue positions in PDE2 are identified, and their replacements in the cAMP-binding GAF-B domain of cyaB1 are shown in parentheses. Colors are blue for nitrogen, red for oxygen, magenta for phosphorous, and white or gray for carbon. Hydrogen bonds are shown in green. See the supplemental table (http://stke.sciencemag.org/cgi/content/full/sigtrans;2003/164/pe1/DC1) for a list of structures and links to their PDB coordinates mentioned in this paper.

are rather diverse [reviewed in (*10*)]. The enzyme activity of cyaB1 and PDE2 is directly stimulated by GAF domain engagement. PDE5 becomes more susceptible to an activating phosphorylation, but is not directly activated by cGMP binding. The affinity of PDE6 for its regulatory subunit is modulated by cGMP binding, but like PDE5, its activity is not directly affect-

ed. These varying effects suggest different structural contacts between the GAF domains and other parts of the enzyme. Indeed, the surfaces of GAF domains that might contact other parts of the proteins that they regulate are not highly conserved, which is also consistent with a multiplicity of regulatory mechanisms. The new result that GAF domains from distantly related proteins can apparently be swapped at will flies in the face of all of this previous thinking about the multiplicity of regulatory mechanisms. It will be both fascinating and challenging to understand this unexpected unity of regulation at the structural level, because this can only be accomplished by determining the structures of full-length GAF domain-containing enzymes.

The GAF domain family is among the largest of all classes of signaling domains appearing in proteins involved in cyclic nucleotide signaling, transcription, phototransduction, and probably many more processes yet to be discovered. The ligands for most GAF domains remain to be identified. In terms of the amount of interest it has received, the GAF domain has been a latecomer compared to other widespread signaling domains. The recent structural and functional results suggest that this is now changing for the better. The chemical and structural biology of GAF domains promises to be an immensely interesting field whose surface has thus far barely been scratched.

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